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Comparative Plasma Proteomics in Muscle Atrophy Induced by Cancer Cachexia and Hindlimb Unloading

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Comparative Plasma Proteomics in Muscle Atrophy
Induced by Cancer Cachexia and Hindlimb Unloading

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Abstract

Introduction: Muscle atrophy results from a dysfunction in protein turnover that leads to loss of mass and function and occurs concurrently with multiple pathologies such as cancer and extended bed rest. Atrophy reduces overall quality of life while increasing morbidity and mortality. Currently, efficacious therapeutic interventions to treat and prevent muscle wasting in all its forms are lacking, however if conserved mechanisms can be identified between wasting conditions, this would aid in the development of multipurpose therapeutics to ameliorate this pathology. **Purpose:** To examine circulating factors present across atrophic pathologies. **Methods:** 35 Male C57BL/6J mice were assigned to hindlimb unloading (HU), Lewis Lung Carcinoma (LLC), or control groups. HU animals were hindlimb-unloaded for 1 week to induce disuse atrophy. LLC cells were injected into the right hind-flank to induce cancer cachexia. Tumors developed for approximately 4 weeks. Age-matched mice were humanely euthanized after experimental intervention. Muscle and plasma were collected for analysis. Plasma was analyzed by tandem mass tag (TMT) proteomics. **Results:** Raw plantaris weight was significantly lower in LLC (22.7%) and HU (18.6%) groups compared to control ($p<0.05$), but not different from each other ($p>0.05$). Equivalent tibia sizes across groups suggest no significant differences in body size. In the LLC-HU comparison, 104 proteins were differentially expressed (DE). 44 were up-regulated and 60 were down-regulated. In the LLC-CON comparison, 91 DE proteins were observed. Of these, 39 were up-regulated and 52 were down-regulated. In the HU-CON comparison, 5 DE proteins were up-regulated and 4 were down-regulated; a total of 9 DE proteins were observed. Several signaling pathways were implicated in this analysis including the acute phase response signaling system and liver X receptor/retinoid X receptor (LXR/RXR) and farnesoid X receptor/retinoid X receptor (FXR/RXR) activation.

Conclusion: Plasma proteomic profiles are altered in cancer cachexia and disuse-induced atrophy. Modulated protein profiles suggest the possibility of multi-tissue involvement in muscle atrophy. Future experiments are required to understand how plasma proteins and muscle interact and to determine the mechanisms of possible novel atrokines observed herein.

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Dedication

I would like to dedicate this thesis to the woman who recognized my passion for research years before I ever did, my late grandmother, Jan Dunlap. Your unwavering love and support in life continues to be a source of motivation and inspiration. May this work and all my future endeavors honor your memory.

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Chapter 1: Literature Review

Muscle Atrophy Overview

Muscle atrophy is a pathological dysfunction in protein turnover where an imbalance in protein synthesis and degradation leads to an overall loss in muscle mass and muscle function *(32).* Atrophy is a serious condition that is often experienced as a side effect of many other diseases including cancer, AIDS, sepsis, muscular dystrophies and chronic inflammatory diseases, and states of disuse such as casting, forced bed rest, or space flight. Unfortunately, atrophy is also exacerbated by drugs intended to treat some of those same diseases, including common chemotherapeutics such as dexamethasone *(26, 47).* Atrophy, also commonly referred to as muscle wasting, increases morbidity and mortality in these patients while significantly reducing quality of life *(45).* Currently, safe and efficacious therapeutic interventions to treat and prevent muscle wasting are lacking. Therefore, the objective of this review will be to examine the different forms of muscle wasting and what factors influence the mechanism of these various etiologies.

Types of Muscle Wasting

Muscle atrophy stems from several etiologies including cancer cachexia, disuse, denervation, dystrophy, sarcopenia, and sepsis. However, skeletal muscle does not respond to these conditions in the same manner. Cancer cachexia occurs secondary to tumors. Patients with gastric and pancreatic cancers are particularly vulnerable to developing cachexia *(21).* These conditions elevate the immune response in the body, creating an inflammatory environment. Cancer cachexia primarily affects type II fibers *(11).* In contrast to cachexia, muscle unloading via forced bed rest, casting, and space flight does not lead to inflammation. In this environment, type I fibers are more susceptible to wasting *(15).* Denervation is an atrophy model that

recapitulates spinal cord injury. The wasting that occurs secondary to denervation is similar to disuse. Inflammation is not induced, and type I fibers are primarily afflicted *(15).* Duchenne's muscular dystrophy is a genetic disorder that disrupts the linkage of the muscle cell's contractile apparatus to the extracellular matrix *(15, 30).* This disorder causes severe muscle damage, inflammation, and premature death *(16).* Fiber types are not discriminated in muscular dystrophy. Sarcopenia is an age-related condition that leads to the loss of muscle mass *(18).* In this condition, inflammation is elevated and, similar to cancer cachexia, type II fibers are the primary target of wasting *(35).* Sepsis is a life-threatening organ dysfunction secondary to a dysregulated host response to an infection *(56).* In order to protect itself from the infection, the body dramatically elevates its inflammatory response *(51).* While this is necessary for survival, it is detrimental to skeletal muscle tissue, particularly type II fibers *(66).* Atrophy occurs under many circumstances and the skeletal muscle exhibits divergent responses to these stimuli. Therefore, the remainder of this review will focus on comparing cancer cachexia and disuse-induced atrophy.

Cancer Cachexia

Cancer cachexia is characterized by a significant loss in body weight due to decreases in muscle mass, with or without fat loss. Cachexia is currently responsible for approximately 20%-40% of cancer deaths *(22).* Patients with gastric or pancreatic cancers are especially prone to cachexia *(23).* It was originally thought that decreased nutritional intake was largely responsible for the development of cachexia. However, current evidence suggests that resolving hypophagia is not sufficient to prevent and reverse cachexia *(29).* This is an extremely complex metabolic disorder in which protein anabolism, protein catabolism, and energy metabolism are all affected.

Protein Anabolism

Significant evidence suggests that protein anabolism is reduced in cachexia *(7, 11, 57, 59, 61, 69).* For example, Smith and Tisdale *(57)* utilized a MAC16 adenocarcinoma injectable cancer model to examine the effect of cancer on protein turnover. The authors implanted mice with a tumorigenic strain of cells. After 10-12 days of implantation, the authors observed palpable tumors. Animals were sacrificed and gastrocnemii were harvested for protein turnover analysis using radioactive labeling techniques. They observed that a body weight loss of 15%-30% corresponded to a 60% decrease in protein synthesis. Similarly, Samuels et al. *(50)* utilized a C26 adenocarcinoma cancer model to examine the effect of cancer cachexia and chemotherapy on protein synthesis and degradation. The authors employed the flooding dose method to measure protein turnover *in vivo*. They found that after the onset of cancer cachexia, mice exhibited significant reductions in protein synthesis. The incorporation of the chemotherapeutic agent cystemustine rescued protein synthetic rates in tumor bearing animals. At this point, there is a consensus that protein synthesis is down regulated in cancer cachectic subjects.

This decrease in protein synthetic rate is likely due to the down-regulation of several anabolic factors including insulin-like growth factor 1 (IGF-1), Akt, mTOR, and satellite cells *(22, 58).* IGF-1, Akt, and mTOR are connected in a prominent PI3K/Akt protein synthetic pathway. In healthy conditions, insulin binds IGF-1 which leads to a conformational change in the receptor and phosphorylates intracellular IRS-1. Activated IRS-1 causes a cascade of phosphorylation events that activates Akt and leads to the activation of mTOR. Phosphorylated mTOR is associated with a dissociation of binding protein 4E-BP1 from the eukaryotic initiation factors 4G and 4E that form a scaffold and bind to the 5’ region of mRNA. This dissociation allows for the binding of 4A and the formation of the 4F complex that allows for ribosomal

recruitment and mRNA translation *(55).* A down-regulation of any of these factors will negatively influence protein synthesis. Satellite cells are unipotent stem cells necessary for muscle maintenance, repair, and hypertrophy *(20).* When signaled by damage, satellite cells proliferate, differentiate, and migrate to the site they are required. A decrease in the available pool of satellite cells limits muscle growth and repair. A perturbation in any of these anabolic factors will limit or reduce overall protein synthetic rates in afflicted muscle.

Protein Catabolism

The available literature suggests that protein catabolism is elevated in cancer cachexia *(11, 60, 62, 69,72).* Smith and Tisdale *(57)* observed a significant increase in protein degradation with a 16%-20% weight loss. They measured protein degradation *in situ* in the gastrocnemius via radioactive labeling techniques. They reported a maximal increase of catabolism at 240% from healthy conditions when weight loss was at 30%. Additionally, Samuels et al. *(50)* observed significant increases in protein degradation at the onset of cachexia. Their chemotherapeutic agent did not increase this effect. It is worth noting that they measured degradation indirectly by extrapolating the relationship between growth and synthesis. Studying protein degradation is difficult, but there seems to be a consensus that catabolism is significantly elevated under cachectic conditions *(32).*

This elevation in degradation may be due to the up-regulation of several catabolic markers including cytokines, myostatin, forkhead box O (FoxO), reactive oxygen species (ROS), nuclear factor (NF-κB), and proteolysis inducing factor (PIF) *(22, 58).* NF-κB is one of the primary transcription factors that transduces (tumor necrotic factor) TNF-α signals into cells. NF-κB is also responsible for activating the gene expression of other cytokines. Together, tumor factors and cytokines up-regulate the ubiquitin-proteasome system, promoting protein

degradation. FoxO transcription factors also play a major role in catabolism. In healthy conditions, FoxO is phosphorylated by PI3K/Akt and is inactivated. In its active conformation, FoxO translocates to the nucleus and up-regulates the expression of skeletal muscle specific E3 ubiquitin ligases muscle RING-finger protein-1 (MuRF1) and atrogin-1/muscle atrophy F-box (MAFbx). FoxO also up-regulates the expression of other autophagy related genes LC3 and Bnip3. The FoxO system is influenced by ROS, PIF, and IGF-1, all of which are modulated by cachexia. PIF plays an additional role in catabolism as it is shown to inhibit mRNA translation via phosphorylation of the eukaryotic initiation factor 2α. Myostatin also contributes to cachexia's catabolic effect. Myostatin is a well-known protein responsible for negatively regulating muscle mass. In healthy conditions, myostatin expression is regulated by IGF-1. However, due to the down-regulation of IGF-1 in cachexia, myostatin can be overexpressed, attenuating muscle growth and contributing to a loss in muscle mass *(58).* Several pathways are involved in protein catabolism. The pathologic modulation of these pathways contributes to an undesired up-regulation of protein degradation.

Energy Metabolism

Mitochondria are crucial for energy metabolism in skeletal muscle. However, it is not enough to consider mitochondria as the 'powerhouse of the cell" without also considering mitochondrial quality which relates to the overall health and function of the mitochondrial network. An impaired mitochondrial network produces less ATP and significantly more reactive oxygen species (ROS) which is detrimental to cell health (*1*). The mitochondria regulate network quality through various processes. These include the biogenesis of new mitochondrial materials, the incorporation of new material and removal of dysfunctional mitochondria via fusion and

fission (dynamics), and the autophagic removal of mitochondria that have been removed from the network (mitophagy) *(49, 73).*

Evidence suggests that the mitochondrial network is impaired in cancer cachexia and may play an initiating role *(10, 33, 43, 44, 64).* Brown et al. *(10)* utilized the $Apc^{Min/+}$ genetic model for colorectal cancer and a Lewis Lung Cancer (LLC) injectable cancer model to understand the cellular events that occur in the development of cancer cachexia. A cachectic phenotype was observed 4 weeks post-injection in the LLC animals. However, mitochondrial respiration was significantly decreased at 3 weeks and ROS production was significantly increased at 1 week. We electroporated the right flexor digitorum brevis with the fluorescent reporter gene pMitoTimer to observe mitochondrial quality via fluorescence microscopy *(36).* In the presence of this reporter protein, healthy mitochondria fluoresce green while unhealthy mitochondria oxidize the protein and fluoresce red. This allows the red:green ratio in these images to be analyzed to determine mitochondrial quality. At 2 weeks, mitochondrial quality was compromised in these animals. Marzetti et al. *(38)* performed a study in human cancer cachectic patients and observed perturbations in gene expression related to the regulation of mitochondrial quality. The authors performed muscle biopsies on the rectus abdominus of their subjects (control, cancer, cancer cachexia groups). They observed that fusion protein Mfn2 was down-regulated in both cancer groups regardless of cachexia diagnosis. However, they found that the fission protein Fis1 was significantly up-regulated only in the cachexia cohort. They did not note any significant differences in the expression of the master regulator of biogenesis, PGC-1α. Interestingly, mitophagy proteins PINK1 and Parkin were significantly down-regulated in both cancer conditions which may suggest impaired or failed mitophagy. Remarkably, the upregulation of Fis 1 corresponds to observations made by Brown et al. *(10)* in LLC cachectic

mice. Regardless of species, there is evidence that degeneration of mitochondria is involved in the pathogenesis of cancer cachexia.

Disuse-Induced Atrophy

Disuse-induced atrophy is common in forced bed-rest, limb casting, and space flight *(4).* In these conditions, atrophy is associated with the lack of muscular tension, or unloading. Whether temporary or prolonged, this disuse can have deleterious effects on force production and increase mortality and morbidity. There are currently no interventions, pharmaceutical or otherwise, available to treat or prevent this disorder. While disuse atrophy is not associated with the inflammation commonly observed in cachexia, protein anabolism, protein catabolism, and energy metabolism are all affected *(48).*

Protein Anabolism

The literature suggests that protein synthetic rates are blunted by disuse-induced atrophy *(34, 48, 54).* Kazi et al. *(34)* examined disuse atrophy in hindlimb suspended C57BL/6J mice. The gastrocnemii were harvested and flooding dose methodology was used to determine protein synthesis following 3 days of suspension. At this time point, muscles had wasted and protein synthesis was significantly decreased. According to their data from DEPTOR knockdown animals, it appears that this decrease in anabolism is likely due to mTOR mediated synthesis inhibition. Similarly, Baehr et al. *(5)* examined the muscle-specific and age-related changes in protein synthesis and degradation in response to hindlimb suspension in rats. The authors were also able to detect wasting in the gastrocnemius after 3 days of suspension. Protein synthesis was assessed using the SuNSET method. Anabolism was decreased in all hindlimb muscles at 3 and 7 days. The synthetic rate in the soleus remained low at 14 days. These data suggest a fiber type dependent response to protein anabolism.

The mTOR pathway is a critical regulatory system for protein anabolism which is negatively impacted by disuse *(4, 54).* Specifically, Kazi et al. *(34)* suggest that knocking down DEPTOR, an mTOR inhibitor, can rescue protein synthesis in hindlimb unloading. It is known that acute immobilization can alter the expression of more than 600 mRNAs, but their exact effects remain essentially unexplored. Promising targets have emerged from this data. Two transcription factors, activating transcription factor 4 (ATF4), and p53 are up-regulated in atrophy. These proteins are shown to be sufficient in the generation of atrophy. Additionally, p21, an inhibitor of cyclin-dependent kinases is up-regulated in immobilization induced atrophy. Downstream from p21 is spermine oxidase. It appears that the up-regulation of p21 leads to the inhibition of spermine oxidase and, in turn, atrophy *(4).* These discoveries are potentially impactful in developing treatments for atrophy. However, more work is needed to determine the mechanism through which these proteins act on skeletal muscle in wasting conditions. Though the data do not completely agree, it seems that protein synthesis is blunted to a degree in most tissues upon unloading.

Protein Catabolism

Baehr et al. *(5)* examined both protein synthesis and degradation. They indirectly observed catabolism by analyzing the activities of catabolic markers proteasome and cathepsin L. They suggest that catabolism was increased in the soleus and tibialis anterior muscles of adult rats. They did not observe any changes in catabolic markers in older animals. This suggests an age and fiber type-modulated response to hindlimb unloading. Overall, protein metabolism is affected in unloaded animals. The degree to which anabolism and/or catabolism contributes appears to depend on several factors including age, fiber type, and duration of unloading.

mRNA evidence suggests an up-regulation of the catabolic factors MuRF1, MAFbx, FoxO, and FoxO3a in unloading-induced atrophy *(4).* Insulin resistance will attenuate the PI3K/Akt pathway, leaving FoxO in its active conformation. As previously described *(22, 58),* FoxO will translocate to the nucleus and up-regulate the transcription of E3 ubiquitin ligases MuRF1 and MAFbx. However, it is suggested that the expression of these markers is dependent on the muscle being studied and its time spent unloaded. More work is needed to elucidate the importance of these potential metabolic pathways.

Energy Metabolism

As previously discussed, proper mitochondrial function is imperative to cellular health *(46).* Cannavino et al. *(14)* examined the influence of PGC-1α overexpression on hindlimb unloaded mice. They used C57BL/6J and transgenic mice that overexpressed the mitochondrial biogenesis protein PGC-1α in the skeletal muscle. These animals were suspended for 3, 7, and 14 days. At the given time points, animals were sacrificed and gastrocnemii were harvested for analysis. They found that fusion was impaired in WT animals that had been suspended for 3 days. A change in the oxidative capacity of the muscle suggested impaired mitochondrial function. This dysfunction led to AMPK induced autophagy. They also found that PGC-1α overexpression preserved muscle mass in unloaded conditions. Additionally, overexpression prevented dysfunction of mitochondrial fusion and prevented catabolism via the AMPK pathway. The same laboratory examined the interaction between PGC-1α, mechanical unloading, and the soleus *(13).* Similar to their other findings, hindlimb unloading leads to muscle wasting. In this model, a redox imbalance was observed after unloading. Hindlimb suspension induced catabolic pathways and reduced protein synthetic rates. The authors tried to prevent catabolism in WT animals by treating them with the antioxidant Trolox, a vitamin E analog. They found that

this treatment did not play a preventative role in the induction of catabolic systems. Not surprisingly, oxidative metabolism was impaired after unloading. The authors observed that elevated PGC-1α expression protected soleus CSA, even after mechanical unloading had occurred. This overexpression prevented catabolism without impacting protein synthesis.

These data suggest mitochondrial involvement in disuse-induced atrophy independent of fiber-type. Moreover, the mitochondria represent a significant contributor to and potential initiator of atrophy. This warrants further study of mitochondria as a potential therapeutic target in wasting secondary to mechanical unloading.

Plasma Interactions

Plasma connects the cardiovascular system to every other system in the body. It circulates oxygen introduced by the respiratory system, nutrients absorbed through the digestive system, and hormone signals secreted by endocrine glands to target tissues. Because cancer cachexia is a multi-organ syndrome that affects adipose, liver, heart, and skeletal muscle, there is potential for cachectic muscle to be modulated via circulated factors *(3, 67).* In fact, evidence suggests that the pro-inflammatory cytokine Interleukin 6 (IL-6) is up-regulated in the plasma of cancer cachectic patients *(9, 70).* The value of proteomic profiles in biological fluids as a diagnostic tool and source of potential treatment targets is beginning to be appreciated *(19).* Performing a global plasma proteomic analysis in cancer cachexia would assist this effort and potentially lead to the identification of novel secretory pro-cachectic factors. In addition, it is unclear whether plasma proteomic analysis has been performed in disuse-induced atrophy. It is understood that the mechanism of muscle wasting is dependent on the etiology of the wasting. As such, this analysis would provide novel data that could improve our understanding of the mechanisms in disuse-induced atrophy and potentially provide overarching targets that link the two forms of atrophy.

Purpose

Therefore, the purpose of this thesis will be to examine the differential expression of circulating factors in cachectic and hindlimb suspended models utilizing proteomic methodologies to detect significantly modulated signaling pathways. Doing so will improve our knowledge of muscle atrophy by examining plasma proteomic profiles in pre-clinical models of muscle wasting and elucidating how signaling pathways interact with muscle tissue in atrophic states.

Objectives of Data Analysis

In order to further evaluate proteomic profiles in cancer cachexia and disuse-induced atrophy, this investigation will: 1) describe phonotypes of LLC-induced Cancer Cachexia and Hindlimb Suspended Animal, including body weight and tissue weight, 2) determine the number and identity of significantly modified proteins in plasma for cancer cachectic cohort in comparison to control, 3) determine the number and identity of significantly modified proteins in plasma for hindlimb suspended cohort in comparison to control, 4) determine the number and identity of significantly modified proteins in plasma for hindlimb suspended cohort in comparison to cancer cachectic cohort, 5) determine top pathways impacted by identified proteins in each experimental group, and 6) verify pathway modulation via Western Blot analysis.

Chapter 2: Comparative Plasma Proteomics in Muscle Atrophy Induced by Cancer Cachexia and Hindlimb Unloading

Introduction

Muscle atrophy, commonly referred to as muscle wasting, is a pathological dysfunction in protein turnover where an imbalance in protein synthesis and degradation leads to an overall loss in muscle mass and muscle function *(32).* Atrophy manifests in response to many diseases and disordered states. These pathologic stimuli trigger divergent responses in skeletal muscle. Inflammation—as observed in cancer, sarcopenia, chronic inflammatory disease, and sepsis—predisposes fast-twitch, glycolytic muscle fibers to wasting *(11, 18, 21, 35, 51, 56, 66).* Whereas conditions of disuse, commonly not involving inflammation, such as denervation, forced bed rest, casting, and space flight, primarily affect slower twitch, oxidative fibers *(4, 15, 48).* Patients afflicted by atrophy experience increased rates of morbidity and mortality while their quality of life is significantly reduced *(45).* Currently, safe and efficacious therapeutic interventions to treat and prevent muscle wasting are lacking. To capture the breadth of this pathology, the present study will compare pre-clinical models of cancer cachexia and disuse-induced atrophy.

Cancer cachexia is a multifactorial syndrome associated with an inflammatory microenvironment and characterized by an ongoing loss of skeletal muscle mass, with or without fat loss, that ultimately leads to impaired function *(21).* Cachexia is currently responsible for approximately 20%-40% of cancer-related deaths and patients with gastric or pancreatic cancers are especially prone to this complication *(22, 23).* Because this is a progressive form of wasting that cannot be reversed via nutritional means alone, it is valuable to consider cancer cachexia as a more complex metabolic disorder *(29, 44).* In fact, significant evidence suggests that protein anabolism is reduced in cachexia due to the down-regulation of several anabolic factors

including IGF-1, Akt, and mTOR *(7, 11, 22, 50, 57, 59, 61, 69).* Compounding this issue, the up-regulation of cytokines, myostatin, FoxO, NF-κB, and PIF, coupled with other indirect measures of protein degradation, indicate an increased level of catabolism in cachectic muscle *(2, 11, 22, 50, 57, 58, 60, 69,72).* Taken together, a decrease in protein anabolism and increase in catabolism contribute to a cachectic phenotype. Interestingly, evidence from our laboratory suggests that energy metabolism is also disrupted in cancer cachexia and mitochondrial dysfunction may even play an initiating role in this disease *(1, 33, 40, 43, 49, 64, 73).* Specifically, we observed a decrease in respiration, an increase in ROS production, and compromised mitochondrial network quality prior to the onset of cancer cachexia *(10).* Adding credence to our findings, increased Fis1 content has been corroborated in human studies *(38).* Collectively, evidence suggests many of the primary processes governing muscle mass maintenance are perturbed in cancer cachexia, ultimately shifting the muscle towards a catabolic state indefinitely. Fortunately, the involvement of these pathways presents many potential opportunities for therapeutic targets.

Disuse-induced atrophy, associated with a lack of muscular tension, is a common consequence of forced bed-rest, limb casting, and space flight *(4).* Whether temporary or prolonged, disuse can have deleterious effects on force production and can increase mortality and morbidity *(4).* Although disuse atrophy does not manifest in an inflammatory cellular environment akin to cancer cachexia, dysfunctions in many of the same metabolic processes have been detected *(48).* For example, decreased rates of protein synthesis have been observed in disuse models and, from data suggesting rescued protein synthesis in DEPTOR knockdowns, this observation is likely due to the modulation of the mTOR axis *(5, 34, 48, 54).* In addition, indirect measures of catabolism suggest an increased catabolic rate, likely due to the up-regulation of

ubiquitin-proteasome markers MuRF1, Atrogin-1, FoxO, and FoxO3a *(4, 5, 22, 58).* Interestingly, energy metabolism and mitochondrial function have also been implicated in conditions of disuse *(13, 14, 46).* A reduction in oxidative capacity and decrease in mitochondrial fusion have both been observed *(4, 46).* As in cancer cachexia, these multiple dysfunctions disrupt protein balance and contribute to atrophy while also providing therapeutic opportunities.

Proteomic profiles of biological fluids serve as valuable diagnostic tools and sources of therapeutic targets *(19).* Because cancer cachexia is a multi-organ syndrome affecting multiple organ systems including liver, adipose tissue, heart, and skeletal muscle, there is potential for cachectic muscle to be modulated via circulating factors *(3, 67).* In fact, evidence suggests that the pro-inflammatory cytokine IL-6 is up-regulated in the plasma of cachectic patients *(9, 70).* More so, the development of cachexia in murine models of colorectal cancer is largely dependent on IL-6 *(6).* Though cancer cachexia and disuse-induced atrophy target different muscles and develop secondary to different stimuli, the same metabolic processes are disrupted, providing a potential therapeutic link between the varying atrophic etiologies. To our knowledge, plasma proteomic analysis comparing cancer cachexia and disuse-induced atrophy has not been previously performed. Performing this global plasma proteomic analysis could lead to the identification of novel secretory pro-atrophic factors, provide novel data improving our understanding of the mechanisms in disuse-induced atrophy, and potentially reveal overarching targets linking the two forms of atrophy. Therefore, the purpose of this thesis will be to examine the differential content of circulating factors in cachectic and hindlimb suspended models of muscle atrophy utilizing proteomic methodologies to detect significantly modulated signaling

pathways. Doing so will improve our knowledge of muscle atrophy by elucidating how signaling pathways interact with muscle tissue in atrophic states.

Methods

Animals and Interventions

All animal experiments were approved by the Institutional Animal Care and Use Committees of the University of Arkansas, Fayetteville. A cohort of male C57BL/6J mice were purchased from Jackson Laboratories. The animals were housed in a temperature-controlled room and maintained on a 12:12 h light-dark cycle. The mice were given *ad libitum* access to normal rodent chow and water.

In the present study, we utilized two different pre-clinical models of muscle atrophy—hindlimb suspension to recapitulate disuse-induced atrophy and Lewis Lung Carcinoma (LLC) implantation to induce cancer cachexia. A cohort of control animals was age matched to both experimental groups. All animals were singly housed to match housing requirements of hindlimb suspension.

Lewis lung carcinoma culture and implantation

Tumorigenic cells were prepared as previously described *(10).* Lewis lung carcinoma cells (ATCC CRL-1642) were plated at passage 2. Cells were cultured in 250 mL culture flasks in DMEM supplemented with 10% fetal bovine serum supplemented with 1% penicillin and streptomycin. Once cells reached confluency, they were trypsinized, counted, and diluted in PBS for implantation. Mice were anesthetized with isoflurane and hair was removed from the right hind flank. LLC cells ($1x10^6$) suspended in 100µL sterile PBS were injected subcutaneously into the hind flank of a single cohort of mice at 8 weeks of age as previously described. Tumors developed for approximately 3 weeks. Experimental endpoints were adjusted for excessive tumor

burden. Animals were anesthetized under isoflurane and body weight was recorded. Plantaris, gastrocnemius, soleus, extensor digitorum longus (EDL), tibialis anterior, quadriceps, spleen, epididymal fat, testes, and plasma were quickly collected post-euthanasia. Tissue samples were weighed and snap-frozen in liquid nitrogen for further processing and stored at -80ºC.

Hindlimb Suspension

Skeletal muscle disuse atrophy was induced by hindlimb suspension as previously described *(39, 68).* Mice were subjected to 7 days of hindlimb suspension. Mice were acclimated to handling by researchers for one week prior to unloading. Unanesthetized animals' tails were cleansed with alcohol and betadine, covered with a small amount of benzoin tincture, and dried until the benzoin became tacky. A hair dryer was used to expedite the drying process. A strip of traction tape was wrapped around the base of the tail, just above the hairline. Tape was adjusted so that it did not impede blood circulation and provided an attachment site for the fish-line swivel device connected to the top of the cage. Mice were attached to a hindlimb suspension apparatus designed to allow access to all areas of the cage with only their forelimbs able to contact the cage floor. The unloading device used in our laboratory consists of a single rod that reaches across the cage. The rod has two rubber stoppers to restrain the mouse from moving too close to the cage walls and loading its hindlimbs. The fish-line swivel connects to a pulley system on the rod, allowing the mouse to move with minimal resistance along the rod between the rubber stoppers. The mouse is suspended at an angle of approximately 30 degrees so that the front legs are in contact with the cage floor and the hindlimbs are completely unloaded and unable to reach the floor. The floor is fitted with gridwire to prevent the animal from reloading its hindlimbs with piled up bedding.

A small amount of bedding was placed under the gridwire of each cage to absorb urine and waste. Animals were suspended for seven days and bedding materials were not replaced during the experiment.

Animals were anesthetized under isoflurane and body mass was recorded. Plantaris, gastrocnemius, soleus, EDL, tibialis anterior, quadriceps, spleen, epididymal fat, testes, and plasma was quickly collected post-euthanasia. Tissue samples were weighed and snap-frozen in liquid nitrogen for further processing and stored at -80ºC.

Proteomics

Sample Collection

Whole blood samples were obtained from animals under isoflurane anesthesia. Samples were centrifuged at 3.5 g for 15 minutes. Plasma was pipetted into separate sample tubes and stored at -80ºC. 100 µL plasma was aliquoted and sent to the University of Arkansas Medical Sciences' Proteomics Facilities.

Proteomics Analysis

Tandem mass tag (TMT) proteomics was performed off-site *(41).* Once purified from plasma samples, proteins were reduced, alkylated, and digested using filter-aided sample preparation [Nature Methods 6: 359-62 (2009)]. Tryptic peptides were labeled using tandem mass tag isobaric labeling reagents (Thermo) according to the manufacturer's instructions and combined into multiplex same groups. Normalization was achieved by including a pooled reference sample for each group. Labeled peptide multiplexes were divided into 36 fractions on a 100 x 1.0 mm Acquity BEH C18 column (Waters) using an UltiMate 3000 UHPLC system (Thermo) with a 40 min gradient from 99:1 to 60:40 buffer A:B ratio. This was done under basic pH conditions. Samples were then consolidated into 12 super-fractions. The super-fractions were

further separated by reverse phase XSelect CSH C18 2.5 µm resin (Waters) on an in-line 150 x 0.075 mm column using an UltiMate 3000 RSLCnano system (Thermo). Elution of the peptides was accomplished using a 60 min gradient from 97:3 to 60:40 buffer A:B ratio. Eluted peptides were ionized by electrospray (2.15 kV) followed by mass spectrometric analysis on an Orbitrap Fusion Lumos mass spectrometer (Thermo). MS3 parameters were used for this analysis. MS data was acquired using the FTMS analyzer in top-speed profile mode at a resolution of 120,000 over a range of 375 to 1500m/z. Upon CID activation with normalized collision energy of 35.0, MS/MS data was acquired using the ion trap analyzer in centroid mode and normal mass range. Up to 10 MS/MS precursors were selected for HCD activation with normalized collision energy of 65.0 using synchronous precursor selection. This was followed by the acquisition of MS3 reporter ion data using the FTMS analyzer in profile mode at a resolution of 50,000 over a range of 100-500 m/z. Protein identification and reporter ion quantification was accomplished using MaxQuant (Max Planck Institute) with a parent ion tolerance of 3 ppm, a fragment ion tolerance of 0.5 Da, and a reporter ion tolerance of 0.01 Da. Scaffold Q+S (Proteome Software) was used to verify MS/MS based peptide and protein identifications and to perform reporter ion-based statistical analysis. Protein identifications were accepted if they could be established with less than 1.0% false discovery and contain at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [Anal. Chem. 75: 4646-58 (2003)].

Buffer A = 0.1% formic acid, 0.5% acetonitrile

Buffer B = 0.1% formic acid, 99.9% acetonitrile

Both buffers were adjusted to pH 10 with ammonium hydroxide for offline separation.

Pathway Analysis

Ingenuity Pathway Analysis (IPA; Qiagen, Valencia, CA; http://www.ingenuity.com) software was used for canonical pathway analysis, upstream analysis, and network discovery.

Immunoblotting

Immunoblotting was performed as we have previously described *(10, 28, 37).* Membranes were probed overnight with primary antibodies. Protein targets were selected based on the proteomic data provided by the UAMS Proteomics Core Facilities: SOD3 (R&D Systems AF4817) and Saa (R&D Systems AF2948). Primary antibodies were isolated from goat. Antibodies were diluted in Tris-buffered saline, 0.1% Tween 20 with 5% milk. Membranes were imaged on Protein Simple FluorChem (Minneapolis, MN). Images were analyzed via Alpha View software. All bands were normalized to the 25 kDa band of Ponceau S stain used as a loading control. Plasma was used for immunoblot analysis.

Statistical Analysis

Phenotypic and immunoblot data were analyzed with a one-way ANOVA and Tukey post-hoc test, α set at 0.05. Proteomic data were analyzed with a one-way ANOVA, α set at 0.05. Benjamini Hochberg correction adjusted α to 0.026. For proteomic analysis, differentially expressed proteins (DEP) were identified as surpassing threshold levels of $p<0.026$ and $Log_2FC = 0.6$. All statistical analyses were performed with SAS software.

Results

Muscle atrophy induced by cancer cachexia and hindlimb unloading (Table 1.)

Phenotypic descriptors of applicable body and tissue weights are presented in Table 1. Briefly, when we accounted for tumor mass, total LLC body weight was not significantly different from control or HU ($p>0.05$). Total HU body weight was significantly lower (8%) than

control ($p<0.05$). In both LLC and HU groups, raw plantaris, gastrocnemius, and quadriceps weights were significantly lower compared to control (. $p<0.05$). However, these weights were not significantly different between atrophic conditions ($p>0.05$). Soleus weight was lower in HU compared to control and LLC (33.4% and 23.2%, respectively), while LLC soleus weight was only significantly lower compared to control (13.2%, $p<0.05$). Wet spleen weight, a surrogate marker of inflammation, was ~200% greater in the LLC animals compared to control and HU ($p<0.005$). Equivalent tibia lengths across groups suggested no significant differences in body size.

Global proteomics demonstrates largest differences in LLC-induced cancer cachexia (Table 2.)

Using TMT based discovery proteomics, a total of 369 peptides were identified in the plasma of the animals in this experiment. Following statistical comparison, 5 peptides were similarly modulated between LLC and HU groups and were significantly different compared to CON. Of those, 3 proteins including complement factor 1 (cf1), apolipoprotein B (ApoB), and coagulation factor IX (F9) were found to be higher in plasma from LLC and HU groups compared to CON. Conversely, 2 proteins were found to be lower in plasma from LLC and HU groups compared to CON including complement 7 (C7) and fibulin (Efemp1). However, while the contents of these proteins were statistically significant, they did not meet the Log_2FC criterion to be considered DE. DE proteins discovered in each analysis are presented in Table 2. The comparison of LLC and HU plasma did yield 104 DE proteins. Of these, 44 were up-regulated and 60 were down-regulated in LLC plasma compared to HU plasma. An evaluation of LLC and CON plasma resulted in 91 DE proteins—39 up-regulated and 52 down-regulated—in LLC plasma compared to CON. In the HU and control comparison, 5 up-regulated and 4 down-regulated DE proteins were observed; a total of 9 DE proteins.

Pathway analysis of discovery proteomics (Tables 3-5, Figures 1-4)

Canonical pathway analysis performed by IPA revealed several modulated pathways in each comparison (Tables 3-5, Figures 1-4). Currently, I am presenting the 5 most modulated pathways in each comparison. Commonly modulated processes among comparisons include acute phase response signaling, LXR/RXR activation, and FXR/RXR activation. In the LLC-HU analysis, these pathways were found to be most modulated in conjunction with atherosclerosis signaling and NO and ROS production in macrophages. The LLC-control comparison revealed additional modulations in atherosclerosis signaling and the intrinsic prothrombin activation pathway. Along with the commonly modulated pathways, the HU-control analysis resulted in modulations within the coagulation and complement systems. Most of these modulations do not clearly exhibit activation or inhibition of the pathway. However, IPA was able to predict activity in some these processes. Acute phase response signaling was up-regulated in LLC plasma compared to control and HU plasma (z-score: 2.714, 3.051, respectively). LXR/RXR activation was up-regulated in LLC compared to control and HU plasma (z-score: 0.5, 0.93, respectively). NO and ROS production in macrophages was down-regulated in LLC plasma compared to HU (z-score: -1.0), while intrinsic prothrombin activation was up-regulated in the same comparison (z-score: 0.447).

Many DE proteins implicated in these modulated pathways exhibit predicted activity across multiple pathways and comparisons. 32 DE proteins, 19 up-regulated and 13 down-regulated, were involved in the LLC-HU comparison. 27 DE proteins, 16 up-regulated and 11 down-regulated, were implicated in the LLC-CON comparison. 7 DE proteins, 3 up-regulated and 4 down-regulated, were found in the HU-control analysis. The protein Serum Amyloid A (SAA), implicated in acute phase response signaling, LXR/RXR and FXR/RXR activation, were

involved in every comparison and were the most modulated. Interestingly, SAA responded to atrophy in a divergent manner, being up-regulated in LLC plasma and down-regulated in HU plasma (Log_2FC: 4.17 LLC, -2.16 HU). Apolipoproteins, Fibrinogens, SerpinA3, SerpinF1, PON1, antioxidant proteins, and others were found to be predominantly modulated in LLC plasma. Similarly, SAA, SerpinA1 and Fibrinogens were predominantly modulated in HU plasma.

Immunoblot confirmation of plasma proteomics

Immunoblot analysis was used to confirm plasma proteomics findings. According to our proteomics data, SAA was highly expressed in LLC plasma and repressed in HU plasma. Immunoblot analysis confirmed higher SAA expression in LLC plasma compared to CON. However, because signals were not present in CON or HU samples, we cannot confirm that SAA expression in HU plasma was suppressed compared to CON (Figure 5). This blot was not quantified as SAA signal was only detected in LLC and internal control samples.

Proteomics analysis suggests catalase content was lower in LLC samples compared to HU plasma. SOD3 was probed as an anti-oxidant surrogate to catalase as the molecular weight of catalase overlays albumin, thereby making the catalase signal indistinguishable. Immunoblot analysis did not reveal any significant differences in SOD3 content between LLC plasma and other conditions ($p>0.05$). However, mean SOD3 content was lower in LLC plasma compared to HU and CON (Figure 6).

Discussion

Our study is among the first to compare modulations in plasma proteomes in disuse-induced atrophy and cancer cachexia. Current literature has established that muscle atrophy, secondary to cancer cachexia or disuse, involves several metabolic processes that shifts the

muscle towards a catabolic state *(11, 60, 62, 69, 72).* Additionally, elevated plasma IL-6 levels in cancer cachexia patients suggest a possible link between the plasma proteome and muscle wasting *(71)*. Prior observations made by Baltgalvis et al. *(6)* demonstrated muscle mass was dependent on IL-6 signaling in cancer cachexia development in the $Apc^{Min/+}$ mouse model of colorectal cancer; therefore, circulating factors may be regulating key regulators of muscle mass in atrophic conditions. In an effort to identify atrokines, differing and similar among etiologies of muscle atrophy, the present study compared plasma proteomes across disuse-induced atrophy and LLC-induced cancer cachexia and suggests if muscle mass is a function of circulating factors, conditions are modulated independently of one another. The proteomic profiles predict up-regulated acute phase response signaling, LXR/RXR activation, and intrinsic prothrombin activation and a down-regulation in NO and ROS production in macrophages in LLC plasma. However, the proteomic profiles predict a down-regulation of acute phase response signaling in HU plasma. Moreover, LLC plasma possessed approximately 10 times more DE proteins than HU plasma, further suggesting a greater reliance of cancer cachexia atrophy on the plasma proteome than under conditions of disuse. Our findings present circulating factors as potential atrophic modulators, specifically under cachectic conditions. Furthermore, to our knowledge, we are the first to perform global proteomic analysis in disuse-induced atrophy. The data herein demonstrate the need for continued investigation of plasma's role in influencing muscle in atrophic etiologies.

Modulated pathways observed in atrophic plasma may influence muscle mass

Canonical pathway analysis suggests the involvement of several pathways in muscle atrophy. In all comparisons, acute phase response signaling, LXR/RXR activation, and FXR/RXR activation were modulated. LLC plasma exhibited an up-regulation of acute phase

response signaling LXR/RXR activation and down-regulation of NO and ROS production in macrophages compared to HU. In addition, intrinsic prothrombin activation was altered in LLC plasma compared to control, and the coagulation and complement systems were modulated in HU plasma compared to control. Upstream analyses of these comparisons provided by IPA identifies key proteins affected by the modulations in atrophic plasma and shed light on potential plasma-muscle interactions.

Commonly modulated pathways

Acute Phase Response

The acute phase response is associated with a rapid inflammatory response that can be triggered by many stimuli including neoplastic growth, or tumor development. This response is associated with fever and an increased concentration of pro-inflammatory cytokines, which is largely due to changes in hepatic metabolism (Appendix). In particular, the cytokines IL-6, IL1B, and OSM were predicted to be activated in our analysis, in addition to the STAT3 complex. Several modulated LLC markers observed in our study including down-regulated ALB, APOA1/2, TTR, RBP4, CAT, and up-regulated fibrinogens, SAA, C4A/C4B. APCS, SERPINA1, HPX, HP, SERPINF, ITIH3, and ITIH4 are all consistent with this observation. IL-6 is a known regulator of muscle mass in some forms of cancer cachexia *(6, 70, 71).* It has been suggested to contribute to catabolism by increasing ubiquitin-proteasome activity and reducing activity of anabolic processes *(70).* Moreover, in a C26 cachexia model, Bonetto et al. *(8)* have observed elevated IL-6/STAT3 transcription in skeletal muscle that was associated with the expression and secretion of additional acute phase response proteins into the plasma, suggesting a muscle-plasma interaction and a potential paracrine function of muscle in cachexia. While IL-6 plays a role in cancer cachexia, other modulated proteins identified in this study have exhibited

atrokine activity as well. SAA, the most modulated protein observed here, has been implicated in COPD mediated muscle wasting. Passey et al. *(42)* observed SAA was sufficient to induce atrophy via pro-inflammatory signaling in the Toll-like receptor 2. In addition, under healthy conditions, catalase functions as an antioxidant responsible for managing concentrations of intracellular ROS produced by mitochondria. Selsby et al. *(52)* observed that overexpression of catalase in mice with Duchenne's muscular dystrophy improved muscle function, suggesting improved antioxidant capacity may ameliorate wasting associated with inflammation and excess ROS production. It is worth noting that prior work done in our laboratory suggests that mitochondrial dysfunction precedes wasting in LLC-induced cancer cachexia and in our present analysis, catalase content is lower in LLC plasma compared to CON. These observations make SAA and catalase potential therapeutic targets. In addition, our analysis identified the involvement of various SERPINS. Interestingly, SERPINA3s have been associated with bovine apoptotic control via caspase inhibition which is important for muscle cell survival and myoblast differentiation during repair *(25).* Our analysis revealed that in LLC plasma, some SERPINA3 isoforms are up-regulated while others are down-regulated. This control mechanism presents a unique opportunity and provides us another potential therapeutic target. In HU, our findings demonstrated a down-regulation of this acute phase response pathway, suggesting that disuse-induced atrophy is mediated in a different fashion.

LXR/RXR and FXR/RXR Activation

LXR is a hepatic receptor that forms a heterodimer with RXR and is involved in the regulation of lipid metabolism, inflammation, and cholesterol metabolism. In the up-regulated state, as observed in LLC plasma, the LXR/RXR pathway increases cholesterol metabolism, cholesterol biosynthesis, cholesterol transport, lipoprotein synthesis, lipogenesis, and cholesterol

efflux from hepatocytes (Appendix). In macrophages, up-regulated LXR/RXR is associated with a proliferated immune response. FXR/RXR is related to the LXR/RXR pathway and serves as a regulator of bile acid levels. FXR regulates metabolic processes by linking bile acid regulation with lipoprotein, lipid, and glucose metabolism (Appendix). In our analysis, this regulation of metabolism coincided with a decrease in apolipoprotein content in LLC plasma. Little is known about these pathways' contributions to skeletal muscle mass, but due to overlaps in the DE protein profiles for acute phase response and atherosclerosis signaling, it is possible that LXR/RXR and FXR/RXR modulation may influence skeletal muscle in an indirect manner. To our knowledge, work has yet to be done connecting LXR/RXR and FXR/RXR activity to cachexia, but their contribution to lipid metabolism make them possible influencers of fat mass, a loss of which is commonly associated with the cachectic phenotype, also observed here. While we did see modulations in these pathways in HU plasma, up or down-regulation could not be predicted, suggesting that links between HU and LLC plasma are lacking in these pathways.

Pathways modulated specifically in LLC

In LLC plasma, atherosclerosis signaling, NO and ROS production in macrophages, and intrinsic prothrombin activity were modulated. Atherosclerosis signaling was not found to be up or down-regulated in LLC plasma. Literature suggests that peripheral atherosclerosis can have negative consequences for muscle *(53).* These alterations include increased oxidative stress, increased inflammatory signaling, mitochondrial dysfunction, altered myofiber morphology, fibrosis, ischemia, and apoptosis *(53).* Our findings, while not conclusive in determining the direction of modulation, suggest atherosclerotic signaling has the potential to indirectly contribute to atrophy.

NO and ROS production in macrophages was down-regulated in LLC plasma, due to modulations in proteins such as catalase. The down-regulation of catalase negatively impacts NO and ROS production in macrophages. This decrease in NO and ROS impairs the macrophage's ability to perform standard functions such as responding to infection and neoplasia *(65).* Dysfunctional macrophages may indirectly impact muscle mass. In addition to responding to infection and inflammatory responses, macrophages are needed to debride damaged myofiber debris and secrete regenerative factors that promote the activation and incorporation of satellite cells into areas of muscle damage *(65).* This pathway presents another potential indirect modulator of cancer cachexia.

Intrinsic prothrombin activity was upregulated in LLC plasma. Deguchi et al. *(17)* suggest that myosin can directly influence thrombosis and coagulation of the blood and they observed higher levels of thrombotic factors in cases of acute trauma. Therefore, it appears that our finding of up-regulated prothrombin activity does not enhance atrophy, but instead is likely due to the interaction of inflamed, traumatized muscle and plasma.

Pathways modulated specifically in HU

In HU plasma, we found modulations in acute phase response signaling, LXR/RXR and FXR/RXR activation, the coagulation system, and the complement system. Of these, only the acute phase response signaling pathway was predicted to be down-regulated. Other modulations were deemed neutral. Shifts in the coagulation system were detected. Frigeri et al. *(24)* also made this observation and surmised this result to fluid shifts induced by hindlimb unloading. Therefore, this finding is probably not due to the interaction of plasma and skeletal muscle, but more likely an artifact of our HU protocol. Similarly, the complement system—an aspect of the immune system—was found to be modulated in HU plasma. This system is necessary for the

lysis of foreign cells, clearance of immune complexes, and activation of inflammation and augmentation of the antibody response. Other studies suggest that immune system sensitivity is likely reduced in this condition due to stress *(24, 27)* While coagulation and complement systems serve vital roles, it is unclear whether there is an interaction between these systems, HU plasma, and HU muscle that could exacerbate atrophy. Instead it appears these observed modulations are the result of the stress and fluid shift induced via hindlimb suspension.

Protein modulation in HU

In our analysis, a total 9 DE proteins were detected in HU plasma and of those, 7 were found to be implicated in highly impacted pathways. This small pool of proteins may represent potential circulating atrokines associated with disuse-induced atrophy. The most promising of these targets include Fibrinogen 1 (FN1) and SAA. We observed higher content of FN1 and lower content of SAA in HU plasma compared to CON. Fibrinogen 1 is a marker commonly associated with many diseases and Ueki et al. *(63)* observed that in atrophy induced by burn injury, elevated fibrinogen was associated with increased inflammation and decreased mitochondrial quality. When they used a toll-like receptor inhibiting treatment that impaired fibrinogen's action, mitochondrial health improved, as did burn injury survival rate. Interestingly, in disuse-induced atrophy, mitochondrial dysfunction has also been observed. Increased ROS production, impaired Ca^{2+} handling, and the release of proteolytic activators all promote atrophy, making Fibrinogen 1 a potential therapeutic target *(46).* In LLC plasma, up-regulated SAA contributes to atrophy via increasing IL-6 and TNF-α signaling and increasing induction of Atrogin-1. When exacerbated, IL-6 signaling and Atrogin-1 can have deleterious effects on muscle mass *(42).* However, IL-6 and Atrogin-1 are required for healthy muscle maintenance *(40, 73).* IL-6 is associated with hypertrophic muscle growth and the regulation of

energy metabolism and, in the proper concentrations, Atrogin-1 contributes to mitophagy which allows the mitochondrial network to remove damaged mitochondria and preserve network quality. In our analysis of HU plasma, SAA is significantly down-regulated, possibly suggesting that a threshold of SAA expression is required for muscle mass maintenance.

Conclusions and Future Direction

In summary, this is the first study to investigate a comparison of plasma proteomes in disuse-induced atrophy and cancer cachexia. Several proteins were found to be modulated in atrophic conditions, though more aberrations to the plasma proteome were detected in cachectic compared to disuse atrophy. This observation is consistent with a greater reliance of cachexia on bloodborne signaling as compared to disuse. These DE proteins influenced the activity of many signaling pathways including acute phase response signaling and LXR/RXR and FXR/RXR activation. Up-regulated acute phase response signaling in cancer cachexia likely up-regulates IL-6/STAT3 expression which would cause muscle to secrete additional acute phase proteins, thereby exacerbating cachexia signals. Interestingly, this finding supports a muscle-plasma interaction and highlights muscle as a potential paracrine organ in cachexia. A down-regulation in this same pathway in HU plasma indicates that these etiologies develop independently of each other. Although future studies are warranted to validate these relationships. To accomplish this, future experiments should evaluate proteomic profiles in the muscle to verify predicted muscle-plasma interactions. This study highlights a clear need for the further study of plasma-muscle interactions in muscle atrophy.

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Appendix

Table 1. Body and wet tissue weights at time of harvest in control (CON), hindlimb-unloaded (HU), and Lewis lung carcinoma tumor-bearing mice (LLC). *N* of 10-14/group was utilized. Tibia length was used as an estimate of total body size. Tibia length did not differ between groups; therefore, all tissue weights are presented as non-normalized wet weights. Lettering denotes statistical significance between groups, alpha set at $p<0.05$.

Group	CON (N=10)	LLC (N=14)	HU (N=10)
Body Weight (g)	26.06 + 0.47 a	26.96 + 0.4 a	23.99 + 0.46 b
Tumor (g)	NA	1.84 + 0.18	NA
Body Weight - Tumor (g)	26.06 + 0.47a	25.12 + 0.43a	23.99 + 0.46b
Soleus (mg)	10.07 + 0.25 a	8.74 + 0.27 b	6.71 + 0.25 c
Plantaris (mg)	21.09 + 0.66 a	16.31 + 0.4 b	17.17 + 0.48 b
Gastrocnemius (mg)	131.51 + 1.76 a	119.9 + 2.36 b	114.03 + 2.23 b
EDL (mg)	10.84 + 0.43	9.66 + 0.2	10.48 + 0.48
TA (mg)	49.41 + 1.0 a	44.15 + 0.89 b	46.47 + 0.99 a
Quadriceps (mg)	193.71 + 8.58 a	141.31 + 10.15 b	156.89 + 8.09 b
Spleen (mg)	69.05 + 1.89 a	164.52 + 12.07 b	63.44 + 9.97 a
Gonadal Fat (mg)	532.16 + 39.94 a	351.03 + 16.27 b	289.17 + 20.83 b
Testes (mg)	114.91 + 2.98	109.82 + 2.64	113.09 + 3.46
Tibia (mm)	17.46 + 0.04	17.28 + 0.1	17.49 + 0.04

Table 2. Differentially expressed (DE, statistical significance $p<0.026$, $Log_2FC > 0.6$) proteins found in each comparison. Number of up-regulated and down-regulated DE proteins shown.

Comparison	Up-Regulated DE Proteins	Down-Regulated DE Proteins	Total DE Proteins
LLC-HU	44	60	104
LLC-CON	39	52	91
HU-CON	5	4	9

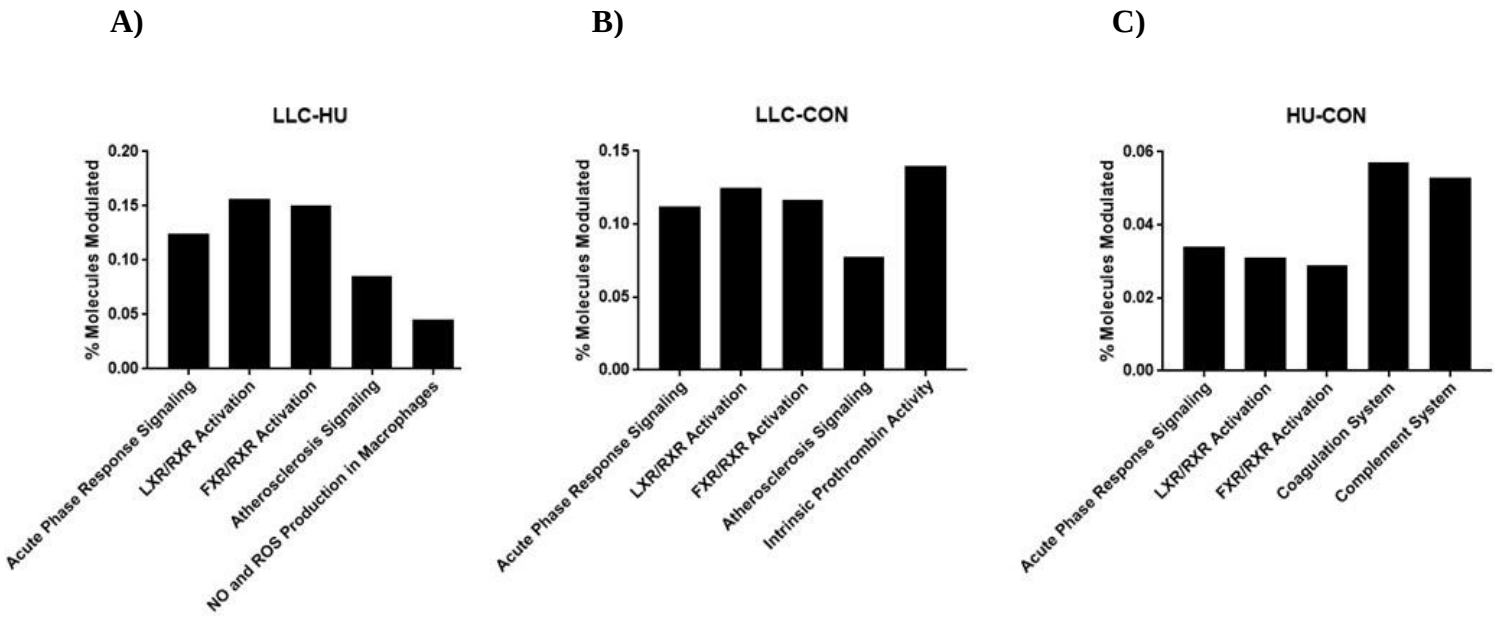


Figure 1. Percent of modulated molecules in the most affected pathways. A) Modulated molecules in the LLC-HU comparison. Z-scores for acute phase response: 3.051, LXR/RXR activation: 0.943, NO and ROS production in macrophages: -1.00. B) Modulated molecules in the LLC-CON comparison. Z-scores for acute phase response: 2.714, LXR/RXR activation: 0.5, intrinsic prothrombin activity: 0.447. C) Modulated molecules in the HU-CON comparison. Z-scores for acute phase response: -1.0.

Table 3. DE proteins involved in the 5 most modulated pathways in the LLC-HU comparison.

Acute Phase Response Signaling	LXR/RXR Activation	FXR/RXR Activation	Atherosclerosis Signaling	Production of NO and ROS in Macrophages
ALB	ALB	ALB	ALB	ALB
AMBP	AMBP	AMBP	APOA1	APOA1
APCS	APOA1	APOA1	APOA2	APOA2
APOA1	APOA2	APOA2	APOC2	APOC2
APOA2	APOC2	APOC2	CLU	CAT
C3	C3	C3	COL1A1	CLU
C4A/C4B	C4A/C4B	C4A/C4B	COL1A2	PON1
FGA	CLU	CLU	ITGB2	RBP4
FGB	FASN	FASN	PON1	SERPINA1
FGG	FGA	FGA	RBP4	
FN1	HPX	HPX	SERPINA1	
HP	ITIH4	ITIH4		
HPX	KNG1	KNG1		
ITH3	PON1	PON1		
ITH4	RBP4	RBP4		
RBP4	SAA1	SAA1		
SAA1	SERPINA1	SERPINA1		
SAA2-4	SERPINF1	SERPINF1		
SERPINA1	TTR	TTR		
SERPINA3				
SERPINF1				
TTR				

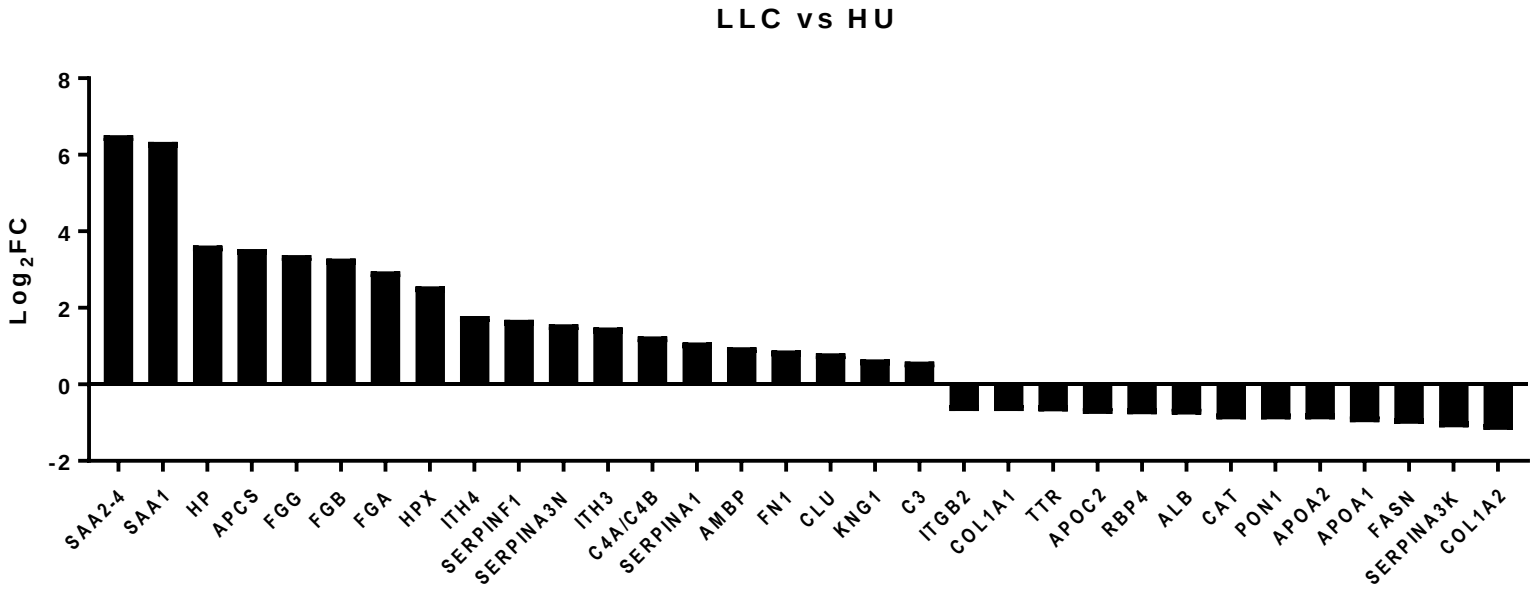


Figure 2. DE proteins involved in the 5 most modulated pathways in the LLC-HU comparison. Log_2FC for each DE in the LLC-HU comparison.

Table 4. DE proteins involved in the 5 most modulated pathways in the LLC-CON comparison.

Acute Phase Response Signaling	LXR/RXR Activation	FXR/RXR Activation	Atherosclerosis Signaling	Intrinsic Prothrombin Activation Pathway
ALB	ALB	ALB	ALB	COL1A1
AMBP	AMBP	AMBP	APOA1	COL1A2
APCS	APOA1	APOA1	APOA2	FGA
APOA1	APOA2	APOA2	APOC2	FGB
APOA2	APOC2	APOC2	CLU	FGG
C4A/C4B	C4A/C4B	C4A/C4B	COL1A1	KNG1
FGA	CLU	CLU	COL1A2	
FGB	FGA	FGA	ITGB2	
FGG	HPX	HPX	PON1	
FN1	ITIH4	ITIH4	RBP4	
HPX	KNG1	KNG1		
HPX	PON1	PON1		
ITIH3	RBP4	RBP4		
ITIH4	SAA1	SAA1		
RPB4	SERPINF1	SERPINF1		
SAA1	TTR	TTR		
SAA2-4				
SERPINA3				
SERPINF1				
TTR				

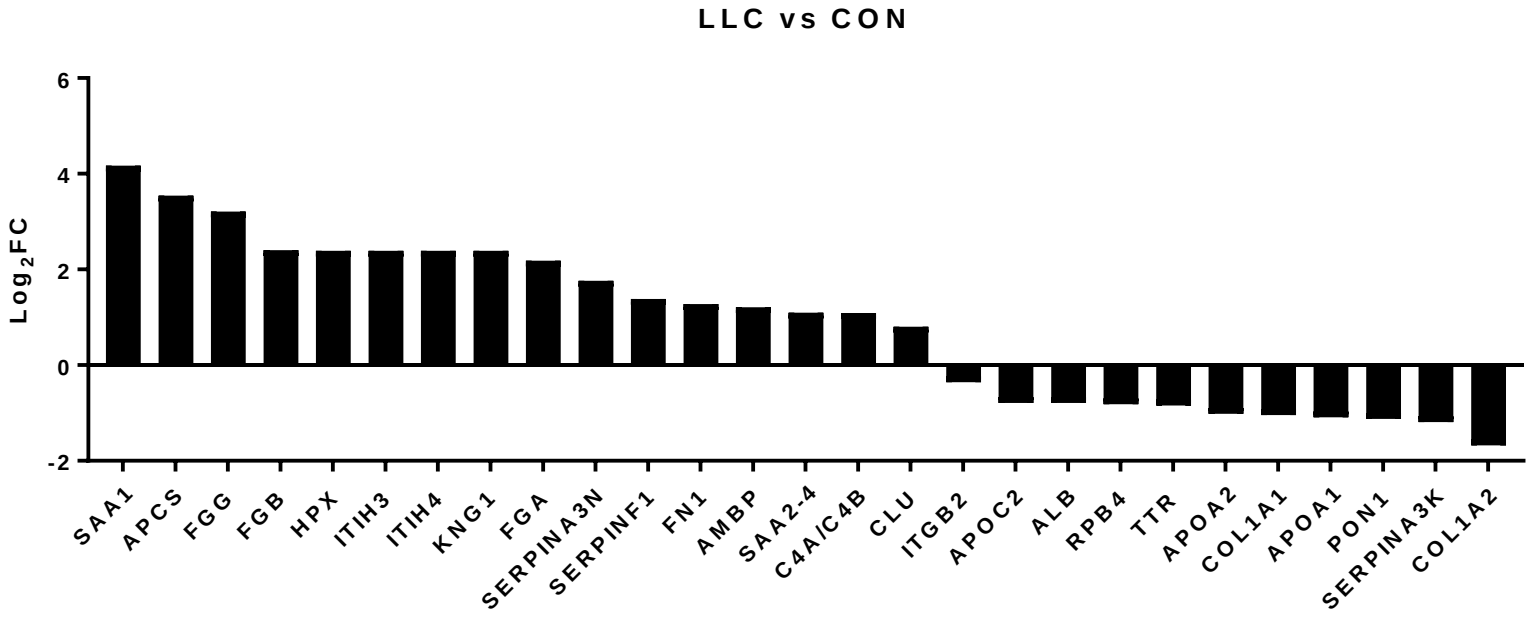


Figure 3. DE proteins involved in the 5 most modulated pathways in the LLC-CON comparison. Log_2FC for each DE in the LLC-CON comparison.

Table 5. DE proteins involved in the 5 most modulated pathways in the HU-CON comparison.

Acute Phase Response Signaling	LXR/RXR Activation	FXR/RXR Activation	Coagulation System	Complement System
FN1	ITIH4	ITIH4	SERPINA1	C7
ITIH4	SAA1	SAA1	FGG	CFH
SAA1	AMBP	AMBP		
AMBP	SERPINA1	SERPINA1		
SERPINA1				
FGG				

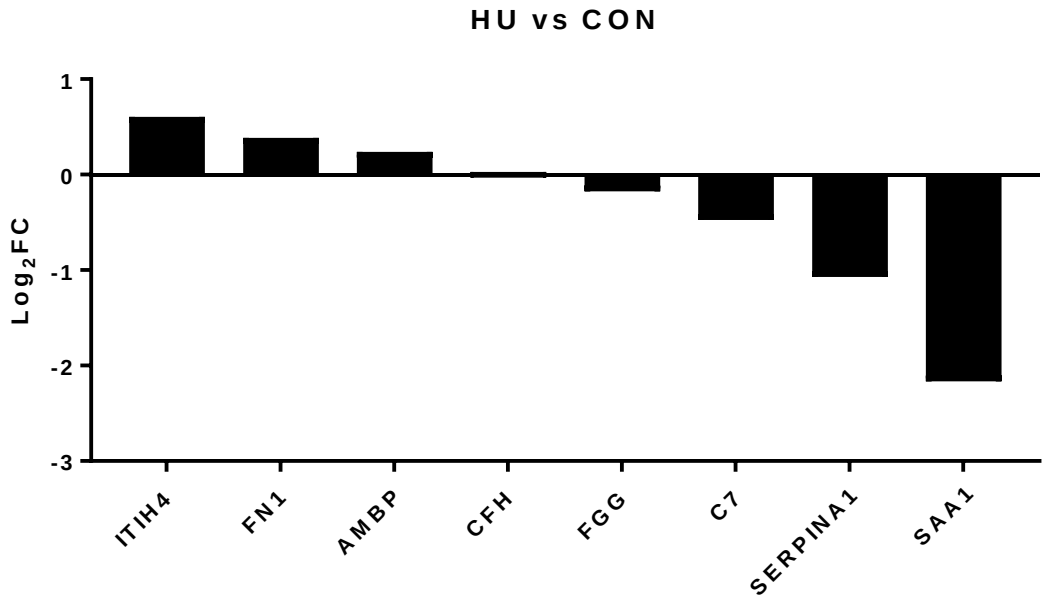


Figure 4. DE proteins involved in the 5 most modulated pathways in the HU-CON comparison. Log_2FC for each DE in the HU-CON comparison.

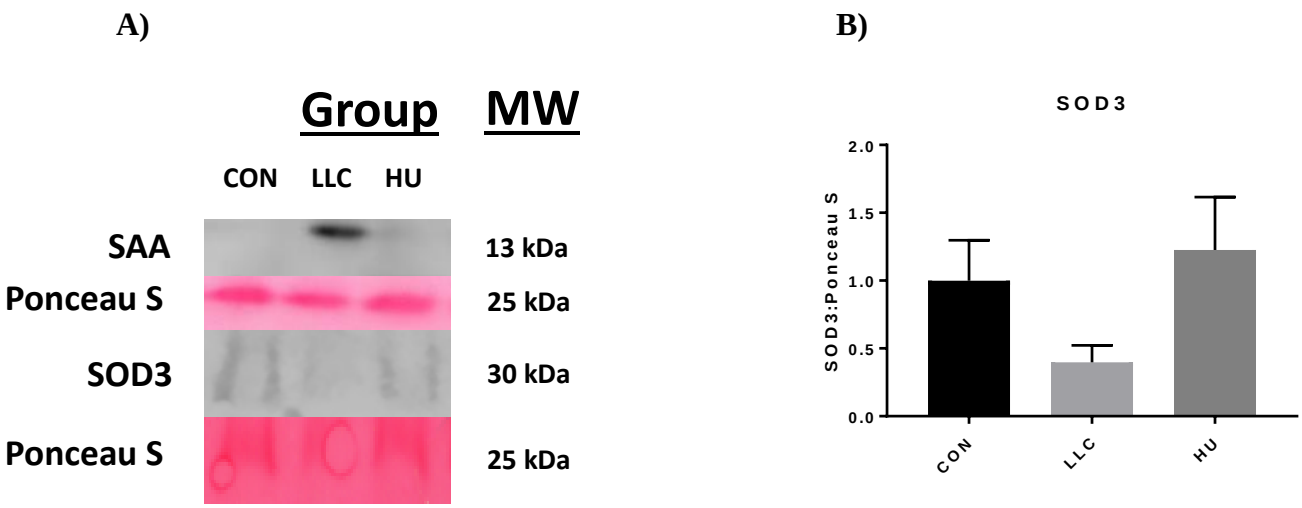
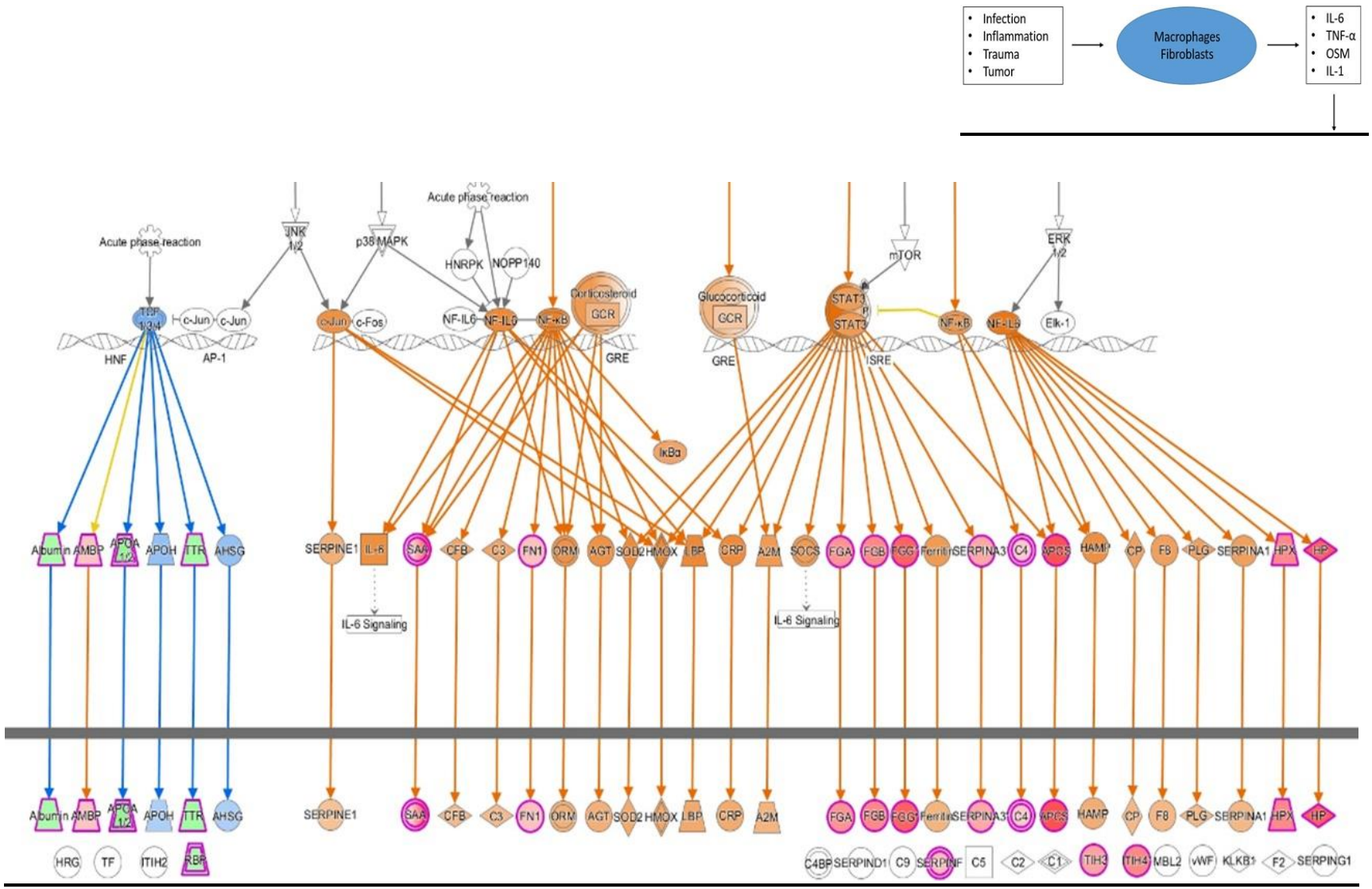
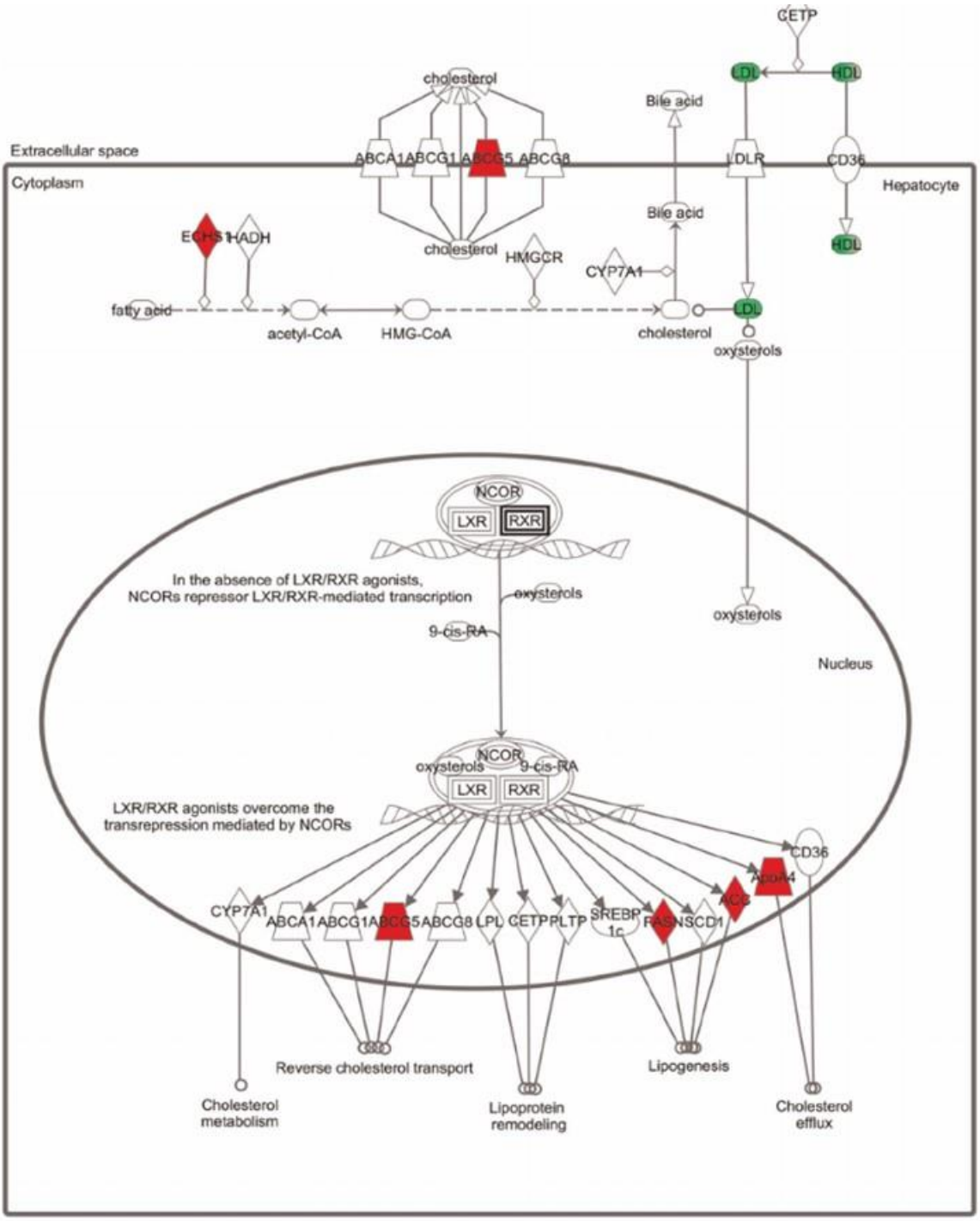


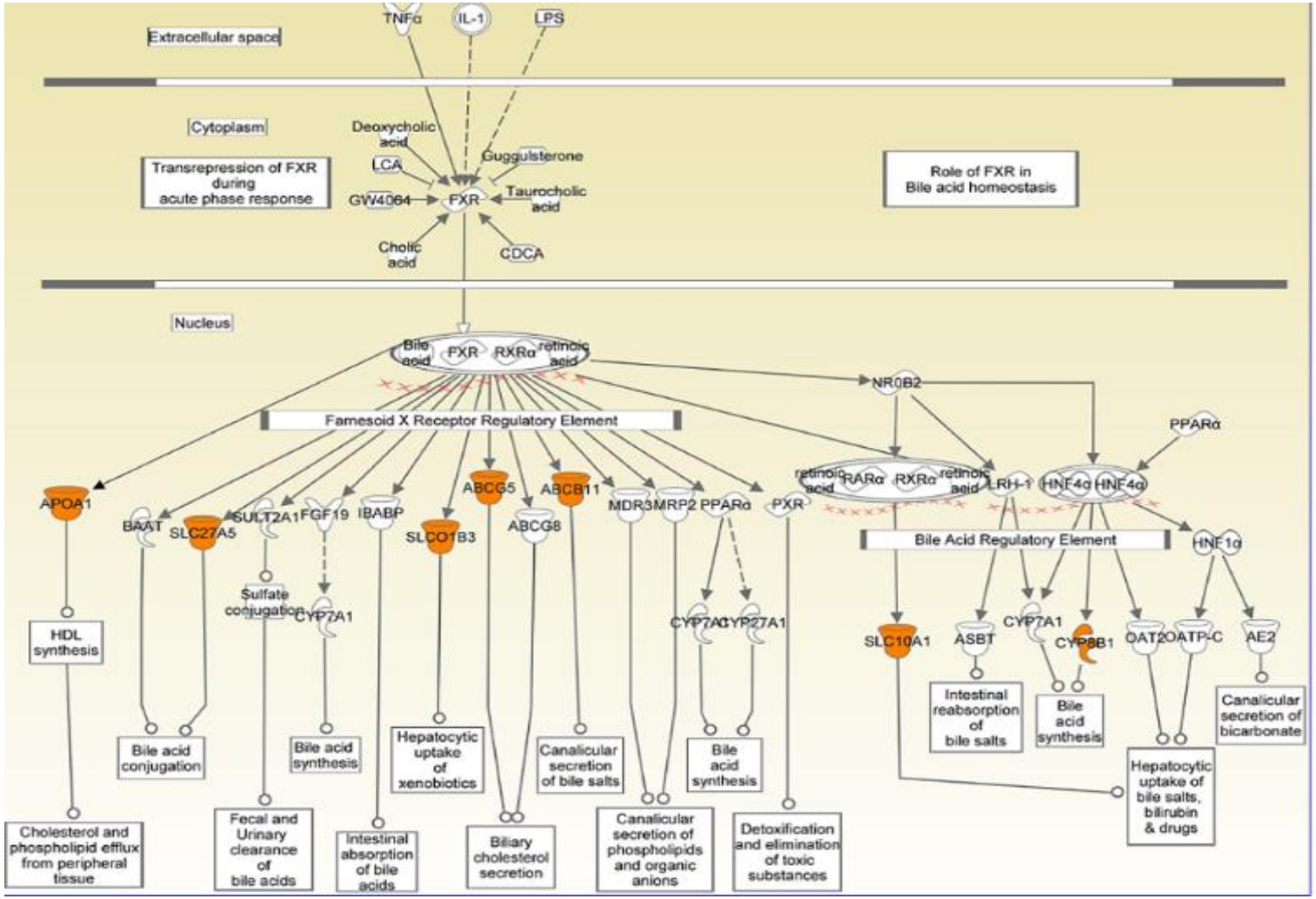
Figure 5. Confirmation immunoblot analysis. A) Representative immunoblot images of SAA and SOD3. B) Quantification of SOD3 blot normalized to Ponceau S stain.



Supplemental Figure 1. Acute Phase Response Signaling in nuclei and plasma. Pathway acquired from IPA.



Supplemental Figure 2. LXR/RXR activation pathway. Pathway acquired from IPA.



Supplemental Figure 3. FXR/RXR activation pathway (Wolf et al., 2013).

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To:	Nicholas Greene
Fr:	Craig Coon
Date:	April 12th, 2018
Subject:	IACUC Approval
Expiration Date:	April 5th, 2021

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **18109:** *A proteomics comparison of disuse and cancer-induced muscle atrophies*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond April 5th, 2021 you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Nicholas Greene, Tyrone Washington, Megan Rosa, and Wesley Haynie. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

UNIVERSITY OF ARKANSAS

Request for Personnel Modification of an Approved Animal Use Protocol (MR)

IACUC Use Only

Protocol number:
Date Received:
Approval Date:
Start Date:
End Date:

Category(s) of animal use:
Agricultural ☐
Biomedical ☐
Field ☐
CITI Training Verified Yes ☐ No ☐

Instructions:

- *This form is required for **any personnel** modifications of an Animal Use Protocol (AUP) which is currently approved by the IACUC.*
- *In completing this MR, briefly state the Objective(s) of the original approved AUP and the qualifications of the proposed personnel modification(s) .*
- *The deadline for getting this form to iacuc@uark.edu is 12:00 Noon on the LAST FRIDAY of every month*

Approved Animal Use Protocol # 18109

Title:
Project Title of Original Protocol: A proteomics comparison of disuse and cancer-induced muscle atrophies

Start and End Dates of Approved Protocol:

Start Date: April 12, 2018 End Date: April 5, 2021

Principal Investigator

Name: Nicholas P. Greene Telephone: 4795756638

Department/Division: HHPR Fax: 4795752853

Campus Mail Address: 321X HPER E-mail: npgreene@uark.edu

Objective:

Objective of the Original Protocol: Compare muscle and blood proteomics with cachectic muscle wasting to disuse muscle wasting.

Objective of the Modification: Add personnel

REQUESTED CHANGES

CITI Training Completed

NAME	DESIGNATION WITHIN THIS STUDY	*Working with the IACUC* (date)	*Minimizing Pain and Distress* (date)	Health Survey Completed (date)	Qualifications & Role in Animal Contact/Handling on this Study
Kirsten Dunlap	Student				Masters student trained by Drs. Greene and Washington. Will aid in animal oversight with assistance
Elizabeth Greene	Other				Experience with small animals for >10 years including husbandry, and multiple surgeries. Will aid in
Seongkyun Lim	Student				PhD student trained by Drs. Greene and Washington. Will aid in animal oversight with assistance from